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14. ABSTRACT

We have completed all proposed training and research project tasks, except a part of Task 4 of research project-related to tumorogenesis experiments. This will be completed during no-cost extension period. This research proposal had two primary objectives which were to (1) increase FIU investigators' research expertise and competitive ability to succeed as independent breast cancer researchers; and (2) to execute research with the promise of identifying molecular causes of breast tumor resistance to anti-estrogen therapy.

We proposed to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our hypothesis is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. We postulate that excess ROS levels induce both *CDC25A* and change p27 phosphorylation promoting the loss of its inhibitory function and leading to antiestrogen resistance. We will investigate whether reducing the oxidative environment of breast cancer cells will restore the anti-proliferative action of tamoxifen and other antiestrogens by repressing *CDC25A* and altering p27 phosphorylation and restoring p27 function.

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INTRODUCTION

This research proposal has two primary objectives which are to (1) increase FIU investigators' research expertise and competitive ability to succeed as independent breast cancer researchers; and (2) to execute research with the promise of identifying molecular causes of breast tumor resistance to anti-estrogen therapy. We proposed to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our hypothesis is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. We postulate that excess ROS levels induce both CDC25A and change p27 phosphorylation promoting the loss of its inhibitory function and leading to antiestrogen resistance. We will investigate whether reducing the oxidative environment of breast cancer cells will restore the anti-proliferative action of tamoxifen and other antiestrogens by repressing *CDC25A* and altering p27 phosphorylation and restoring p27 function.

BODY

THE TRAINING PROGRAM TASKS AND THEIR PROGRESS:

To extend and enhance the FIU investigators' skills in order to increase their research expertise and competitive ability to succeed as independent breast cancer researchers, we proposed to conduct the following training tasks each year during the 4yr period of this project: i) To conduct onsite weekly lab meetings in which FIU investigators and trainees report research data, trouble-shoot, and plan experiments; ii) To meet every other week onsite to facilitate coordination of the project; iii) To participate in the monthly BFBCI Scientist Seminars at the University of Miami in order to broaden FIU investigators knowledge of the most current clinical research in breast cancer; iv) To establish an Invited Expert Breast Cancer Research Seminar Series at the FIU campus; v) To promote breast cancer research at the FIU campus, FIU/BFBCI training program will sponsor an annual onsite Breast Cancer Workshop; vi) To participate in the grant writing workshop entitled "The Molecular Mechanisms of Breast Cancer"; and vii) To prepare a written quarterly progress report of ongoing activities and compile them together to prepare the annual progress report.

In order to meet the objective of training, we have completed all proposed original training tasks. To achieve **Task I**, we have been holding weekly lab meetings on every Monday at 9:30 am at the FIU campus in HLS bldg Rm 596. Since the start date of this grant, both pre-doctoral students and faculty have participated in the weekly lab meetings in which we report the results and from the interpretation of the data we plan experiments for the upcoming week. To achieve **Task II**, all three FIU investigators have been involved in biweekly discussions to facilitate the forward progress of this proposal at FIU. To achieve **Task III**, FIU investigators and pre-doctoral student trainees participated in monthly BFBCI Scientist Seminars at the University of Miami. To achieve **Task IV,** we have been holding the FIU Breast Cancer Seminar Series. To achieve **Task V**, we have held the Annual Breast Cancer Symposiums. To achieve **Task VI**, three faculties participated in NIH grant writing training workshop. This resulted in the submission of several grants to DoD/NIH by all these investigators. To achieve **Task VII**, we have been preparing quarterly progress report of ongoing activities and those reports have been used to prepare annual progress report.

In summary, we have performed all training tasks we proposed to carry out in years 1-4. All tasks have been fully performed and we will continue to maintain this activity during the no-cost period of this award.

RESEARCH PROJECT: We proposed to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our **hypothesis** is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. We postulate that excess ROS levels induce both *CDC25A* and change p27 phosphorylation promoting the loss of its inhibitory function and leading to antiestrogen resistance. We will investigate whether reducing the oxidative environment of breast cancer cells will restore the anti-proliferative action of tamoxifen and other antiestrogens by repressing *CDC25A* and altering p27 phosphorylation and restoring p27 function.

We have completed all proposed research project tasks, except a part of Task 4 -related to xenograft tumorogenesis experiments. Major findings are described in detail below:

1. **Exposure of TAM resistant breast cancer LCC2 cells to 17β-estradiol (E2) and tamoxifen (TAM) produces a rapid increase in ROS levels:** Although the primary mechanism of antiestrogen action is believed to be through the inhibition of ER activation, research over the years has indicated that additional, non-ER-mediated mechanisms exist. Recently, we have shown that the growth of breast cancer cells was inhibited by antioxidants. Our previous findings in MCF-7 cells showed that estrogen-induced ROS promote G1 progression and the early G1 gene *cyclin D1* through signaling phosphorylation of CREB and AP-1 (Roy et al., 2004). Our first objective was to determine the effect of antioxidants on TAM induced ROS in tamoxifen resistant LCC2 breast cancer cells. Before carrying out antioxidant-mediated restoring of tamoxifen sensitivity experiments, we characterized tamoxifen resistant breast cancer cells for their ability to produce ROS in response to E2 and TAM exposure. A DCFH-DA assay was performed using LCC2 cells with or without antioxidants. LCC2 cells respond to E2 and TAM in terms of producing ROS very similar to breast cancer cells. The abilities of these compounds to produce ROS were inhibited by overexpression of catalase (CAT) or manganese superoxide dismutase (MnSOD) or treatment with Ebselen (Fig. 1 and 2).

Fig. 1 Tamoxifen and E2-induced ROS in LCC2 cells is Abrogated by Biological Antioxidants

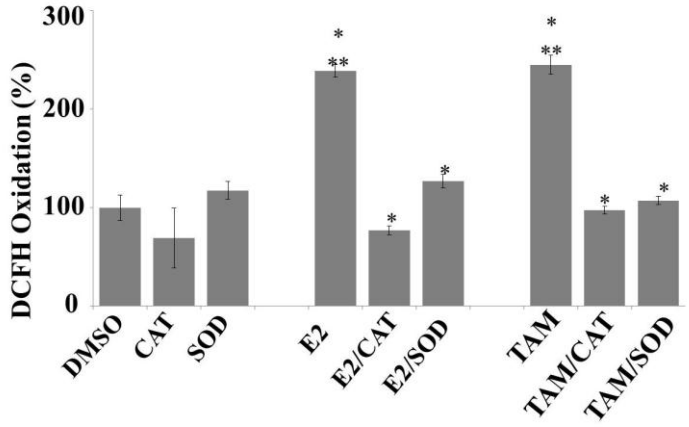
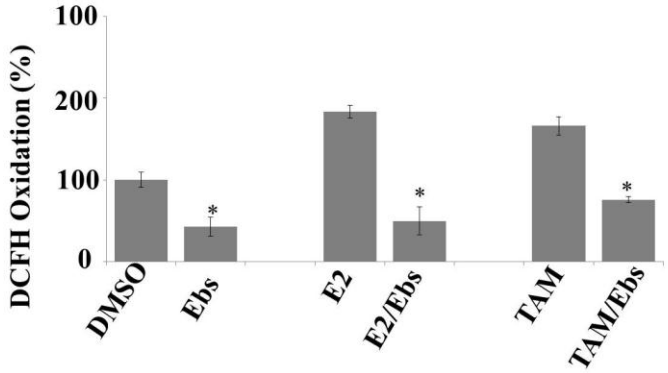


Fig- 2. Tamoxifen and E2 induced ROS in LCC2 cells is Abrogated by Chemical Antioxidant -Ebselen



2. A**ntiestrogen resistant breast cancer cell lines regain sensitivity to inhibitory effects of antiestrogen when oxidant levels are reduced. W**e first confirmed whether E2 and Tam induced oxidants can be reduced by ROS modulators. As shown in **Fig 3** and **4**, ROS production is inhibited in antiestrogen resistant breast cancer

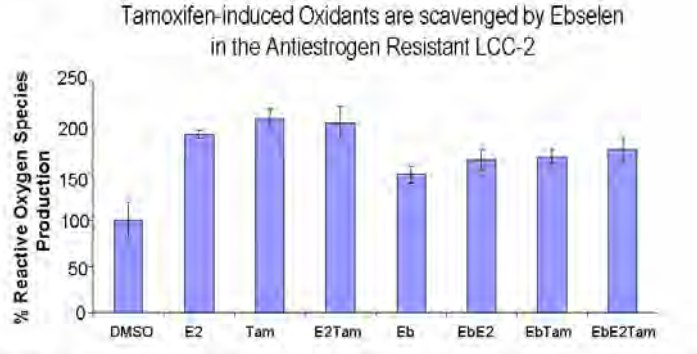


Fig 3 Pre-treatment with antioxidant Ebselen (Eb) 40μM in antiestrogen resistant cell line LCC-2 showed reduction in both 17β-estradiol (E2) and tamoxifen (Tam) induced reactive oxygen species formation. Cells were exposed to 100pg/mL E2, 1uM Tam or a combination of both. Oxidant production was detected with the oxidant sensitive probe DCFH.

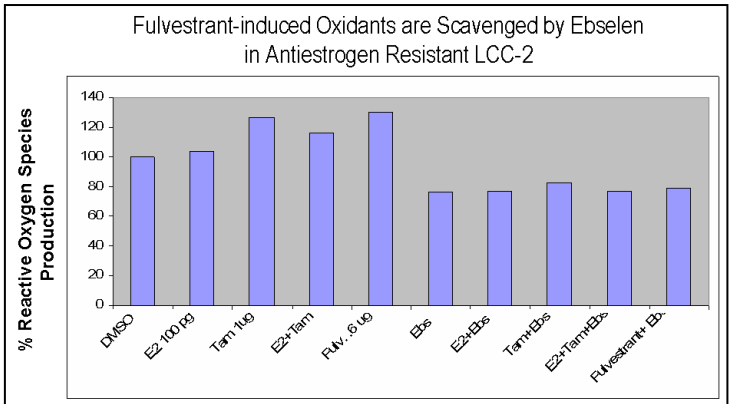


Fig 3B Pre-treatment with antioxidant Ebselen (Eb) 40μM in antiestrogen resistant cell line LCC-2 showed reduction in both 17β-estradiol (E2), tamoxifen (Tam), and Fluvestrant (Fulv) induced reactive oxygen species formation. Cells were exposed to 100pg/mL E2, 1uM Tam, 0.6μg/ml Fulv or a combination. Oxidant production was detected with the oxidant sensitive probe DCFH.

cell line LCC-2 when exposed to ebselen or by overexpression of the antioxidant enzyme MnSOD. Next, we determined whether these same ROS modulators could restore sensitivity of antiestrogen resistant breast cancer cell lines. DNA synthesis was measured using the BrdU incorporation assay. As shown in **Fig 5** and **6**, the

growth inhibitory effect of tamoxifen is regained in LCC-2 cells by treatment with the antioxidant ebselen as well as by the overexpression of the antioxidant enzyme MnSOD.

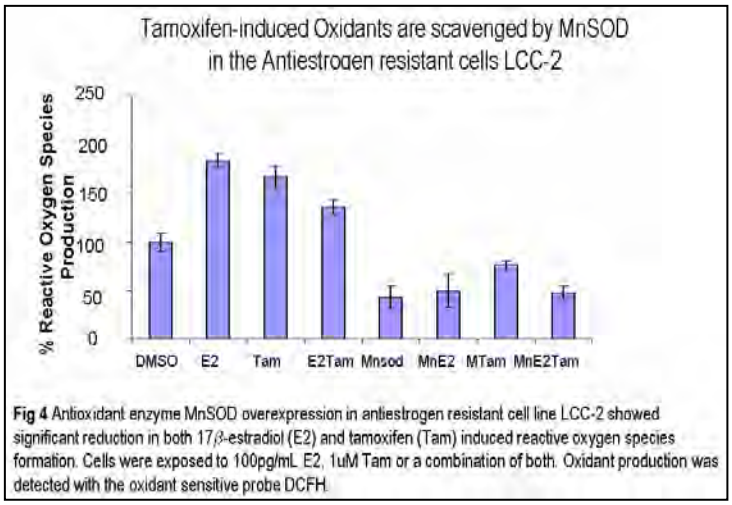


Fig 4 Antioxidant enzyme MnSOD overexpression in antiestrogen resistant cell line LCC-2 showed significant reduction in both 17β-estradiol (E2) and tamoxifen (Tam) induced reactive oxygen species formation. Cells were exposed to 100pg/mL E2, 1uM Tam or a combination of both. Oxidant production was detected with the oxidant sensitive probe DCFH.

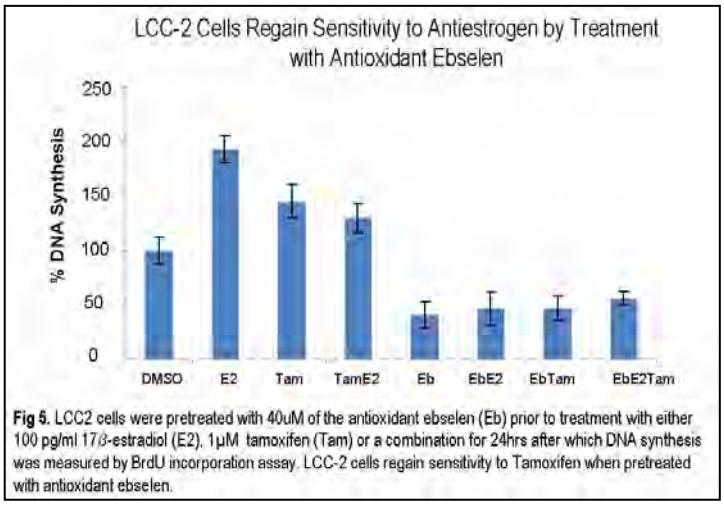


Fig 5. LCC2 cells were pretreated with 40uM of the antioxidant ebselen (Eb) prior to treatment with either 100 pg/ml 17β-estradiol (E2), 1µM tamoxifen (Tam) or a combination for 24hrs after which DNA synthesis was measured by BrdU incorporation assay. LCC-2 cells regain sensitivity to Tamoxifen when pretreated with antioxidant ebselen.

We performed comparative studies with the antiestrogen resistant breast cancer cell line LY-2. A shown in **Fig. 7**, LY-2 cells regain sensitivity to the growth inhibitory effect of antiestrogens Tamoxifen and Fulvestrant when treated with the antioxidant ebselen.

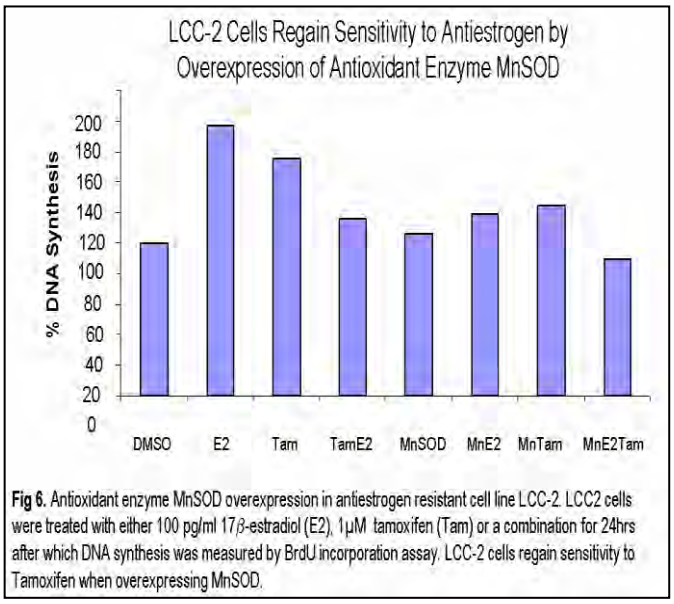


Fig 6. Antioxidant enzyme MnSOD overexpression in antiestrogen resistant cell line LCC-2. LCC2 cells were treated with either 100 pg/ml 17β-estradiol (E2), 1µM tamoxifen (Tam) or a combination for 24hrs after which DNA synthesis was measured by BrdU incorporation assay. LCC-2 cells regain sensitivity to Tamoxifen when overexpressing MnSOD.

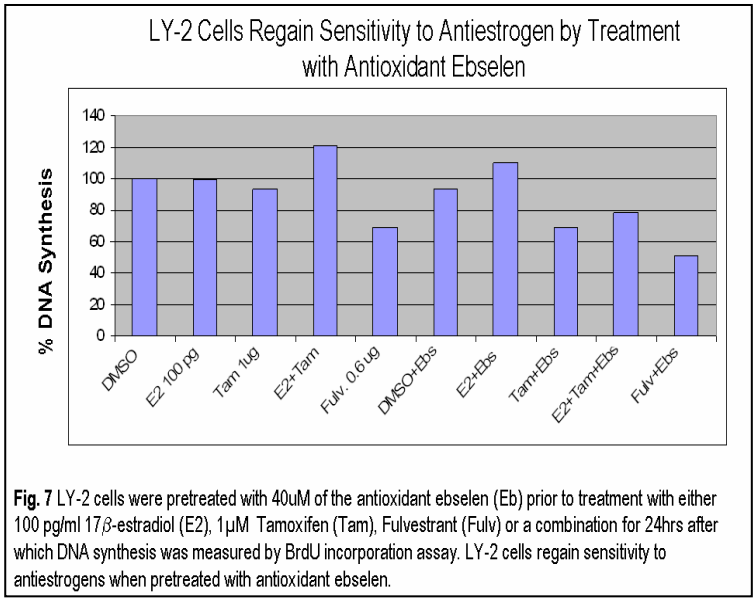


Fig. 7 LY-2 cells were pretreated with 40uM of the antioxidant ebselen (Eb) prior to treatment with either 100 pg/ml 17β-estradiol (E2), 1µM Tamoxifen (Tam), Fulvestrant (Fulv) or a combination for 24hrs after which DNA synthesis was measured by BrdU incorporation assay. LY-2 cells regain sensitivity to antiestrogens when pretreated with antioxidant ebselen.

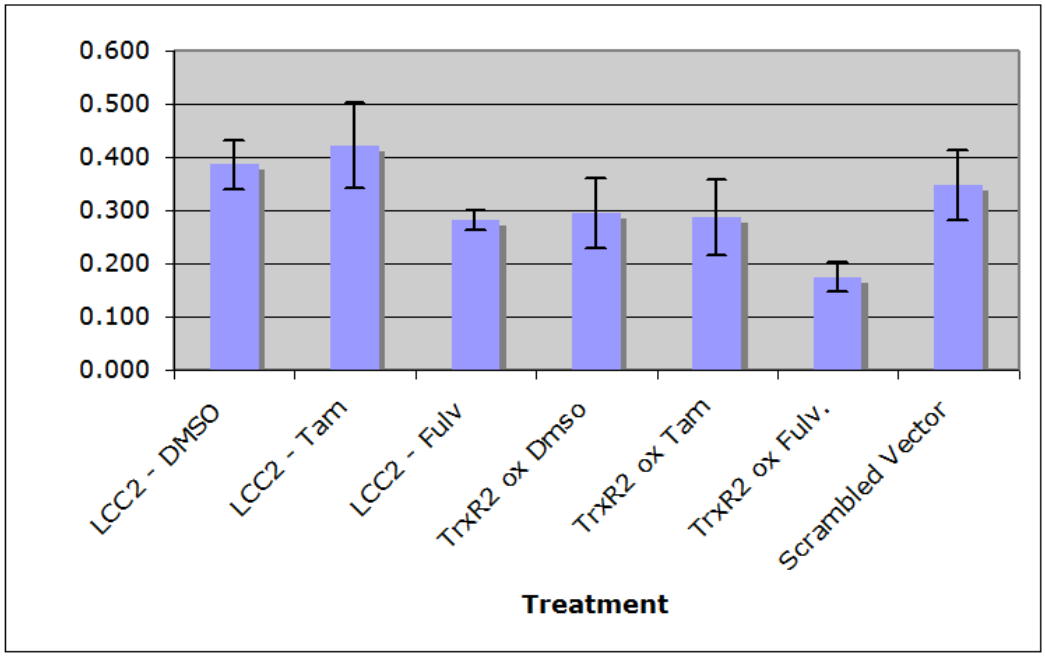
3. **An increase in reduced mitochondrial thioredoxin, through the overexpression of thioredoxin reductase 2 restores the growth-inhibitory actions of tamoxifen in LCC2 (tamoxifen resistant) breast cancer cells**. The purpose of this experiment was to explore the role of TrxR2 on DNA synthesis of tamoxifen resistant cells. As with the previous experiment, we are altering the oxidative state of tamoxifen resistant breast cancer cells through increasing the expression of TrxR2. This, in turn reduces the antioxidant thioredoxin 2 to its active form, and reduces the oxidative state of the cells. Since tamoxifen is known to increase the oxidative state of breast cancer cells, an increase in reduced mitochondrial thioredoxin, through the overexpression of thioredoxin reductase 2, should restore the growth-inhibitory actions of tamoxifen in LCC2 (tamoxifen resistant) breast cancer cells, and thus decrease DNA synthesis.

As shown in **Fig.** 8, the antiestrogen resistant LCC2 breast cancer cells normally grow in the presence of tamoxifen, however, in cells overexpressing TrxR2 we observed the LCC2 cells to regain their sensitivity to the growth inhibitory effects of tamoxifen. There was a 33% decrease in DNA synthesis in TrxR2 overexpressing cells exposed to tamoxifen indicating that when the oxidative state of tamoxifen resistant cells is reduced, the tamoxifen inhibits DNA synthesis.

Fig. 8

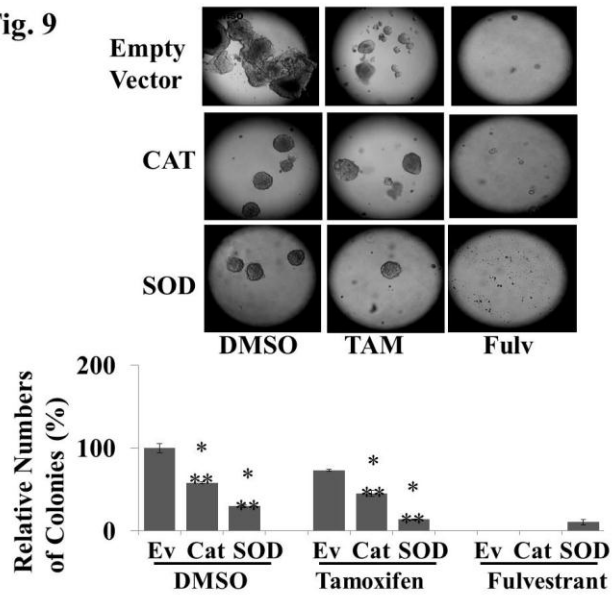
TrxR2 Overexpression - BrdU

	LCC2 - DMSO	LCC2 - Tam	LCC2 - Fulv	TrxR2 ox Dmso	TrxR2 ox Tam	TrxR2 ox Fulv.	Scrambled Vector
10 min	0.442	0.498	0.306	0.235	0.197	0.186	0.421
	0.401	0.43	0.267	0.36	0.344	0.165	0.262
	0.347	0.449	0.281	0.338	0.34	0.14	0.356
	0.351	0.311	0.269	0.239	0.26	0.204	0.348
mean	0.385	0.422	0.281	0.293	0.285	0.174	0.347
Std Dev.	0.045	0.079	0.018	0.065	0.070	0.028	0.065
% change	100	110	73	76	74	45	90



4. Anchorage independent growth assay also showed that antiestrogen resistant breast cancer cell lines regain sensitivity to inhibitory effects of antiestrogen by treatment with ROS modifier. While LCC2 cells normally grow tumors in soft agar in the presence of tamoxifen, we would expect that the overexpression of ROS modifier TrxR2, catalase or MnSOD, or antioxidant treatment would cause tamoxifen to regain its growth inhibitory effects in this antiestrogen resistant cell line. To determine the effect of ROS modifiers on anchorage-independent growth in TAM resistant cells, a colony assay was performed using LCC2 cells. Anchorage independent growth was significantly inhibited by Fulvestrant but not by TAM or DMSO. Fulvestrant was included in this experiment as a positive control because LCC2 cells are sensitive to it. As shown here in Fig. 9,10 and 11, Ebselen pretreatment or the over expression of

Fig. 9



CAT or MnSOD or TrxR2 in TAM resistant LCC2 cells significantly inhibited anchorage independent growth compared to EV infected or uninfected cells. Conclusions from this experiment is that ROS generated from tamoxifen treatment is necessary for the growth inhibition of antiestrogen sensitive breast cancer cells. These data supports our hypothesis because the manipulation of the oxidant state of antiestrogen resistant cells, increased their sensitivity to the growth inhibitory effects of tamoxifen. Thus, it appears that antiestrogen resistant breast cancer cells have adapted to a higher level of oxidant stress which can be reversed by the overexpression of ROS modifier or antioxidant treatment.

Fig. 10

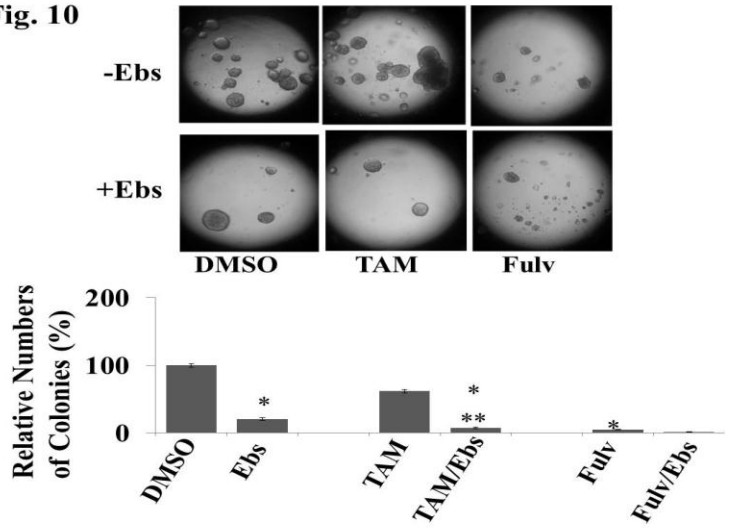
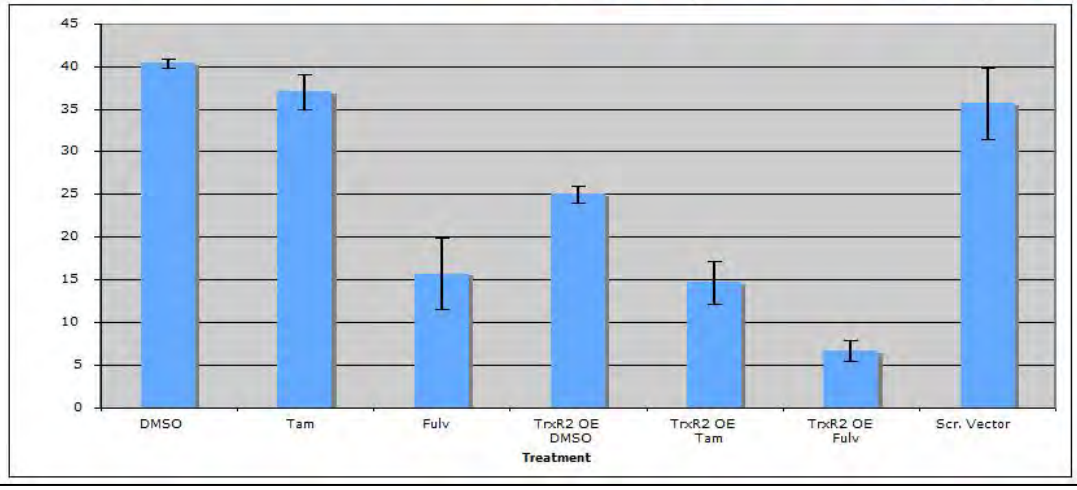


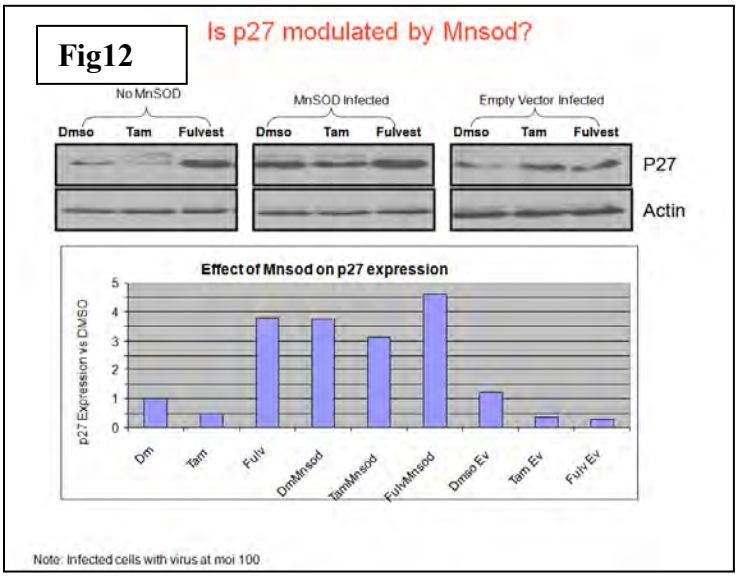
Fig. 11

TrxR2 Overexpression - Soft Agar

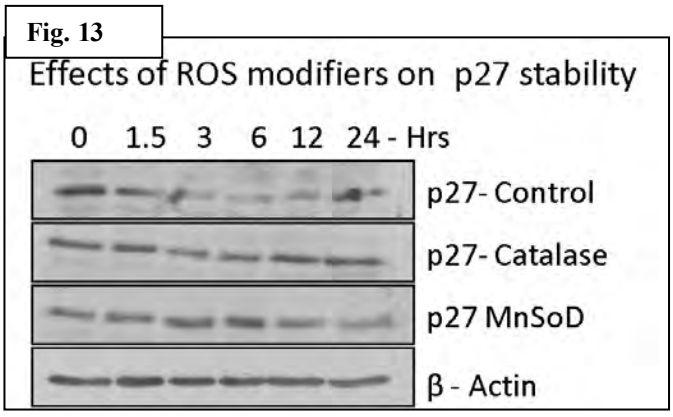
LCC-2 cells							
TrxR2 OX							
Soft Agar Day 23							
12-Mar-09	DMSO	Tam	Fulv	TrxR2 OE DMS	TrxR2 OE Tam	TrxR2 OE Fulv	Scr. Vector
plate 1	40	35	11	25	17	6	37
	40	37	19	24	12	6	31
	41	39	17	26	15	8	39
mean	40	37	16	25	15	7	36
Std. Dev.	0.58	2.00	4.16	1.00	2.52	1.15	4.16



5. **Cell cycle regulator, p27, ise critical to cell cycle effects of redox manipulations, after cell treatments with ROS modifiers and antiestrogen.** It is expected that redox signaling inhibition by pharmacological and genetic approaches restores the cell cycle inhibitory function of p27 and enhances sensitivity of resistant breast cancer tumor cells to tamoxifen or fulvestrant. These studies would confirm that adaptation to higher oxidative state in breast carcinoma is not only causally linked to antiestrogen therapy resistance, but also elucidate the mechanism thereof.We first determined the effect of ROS modifiers catalase and MnSOD had on the protein level of p27 in LCC2 (Tamoxifen resistant) breast cancer cells exposed to antiestrogens. As shown in **Fig.** 12 , overexpression of MnSOD showed a pronounced increase in p27 levels compared to control. Similarly, tamoxifen exposed LCC2 cells overexpressing catalase also showed an increase in the p27 level. This data supports the overall hypothesis because it shows that p27 levels are restored when exposed to tamoxifen. Thus, the increase in p27 levels suggest that by decreasing the oxidant state of antiestrogen resistant breast cancer cells restores redox signaling of p27 by tamoxifen. The increase in p27 levels most likely will inhibit cyclin dependent kinase activity and ultimately contribute to the suppression of the cell cycle.



6. **The overexpression of catalase prevented the degradation of p27**. Since the activity of p27 is regulated by degradation, we next determined the effect of ROS modifiers - catalase and MnSoD on the stability of p27 by looking at p27 expression. **The purpose of these experiments** was to determine whether a shift from a pro-oxidant to a more reduced state can restore the cell cycle inhibitory function of p27 by increasing the stability of p27 or preventing p27 proteolysis. If a pro-oxidant cell environment decreases p27 stability, then we expect to see increased p27 stability in response to catalase overexpression. Catalase overexpression is expected to lower the level of intracellular hydrogen peroxide shifting the cell to a more reduced redox state.



As shown in Fig. 13, p27 levels decreased with time in wild-type LCC2 cells (control), whereas the overexpression of catalase prevented the degradation of p27. This correlates with the increase in p27 levels we observed in breast cancer resistant cells that regain their sensitivity to tamoxifen. The decrease in p27 in untreated wildtype LCC2 cells implies that reducing the oxidant state of the cell prevents degradation of p27. The presence of p27 will contribute to the inhibition of cyclin dependent kinase and ultimately inhibit cell cycle progression. These data partly explain the regained tamoxifen sensitivity of antiestrogen resistant breast cancer cells. In specific, the more reduced intracellular environment will restore the level of p27 and its contribution to the regulation of the cell cycle. Since tamoxifen is known to inhibit breast cancer cell growth via its effects on p27; by restoring the level of p27 in LCC2 cells will make it vulnerable to tamoxifen once again.

7. **ROS-mediated activation of Akt is critical to ROS activation of G1 cyclin-Cdks and the loss of Cdk2 inhibitory function of p27 in resistant cells**. It is known that Akt phosphorylates Thr 157 of p27 and this reduces the nuclear import activity of p27. If p27 is not in the nucleus, then it cannot inhibit G1 regulators of the cell cycle. We measure the expression of PI3K and its downstream target –AKT in catalase or MnSOD overexpressing LCC2 cells exposed to tamoxifen. We expect that AKT would either increase or remain the same in LCC2 cells exposed to ROS modifier-catalase plus tamoxifen.

As shown in Fig. 14, when comparing catalase overexpressing LCC2s to control, there is a dramatic increase in AKT phosphorylation from tamoxifen exposure. The overexpression of MnSoD (Mn) or ebselen (Eb) co-treatment did not produce any effect on the phosphorylation of AKT or PI3K. As expected the level of total AKt was not changed by the treatment. The increase in AKT phosphorylation implies that p27 is phosphorylated more at Thr-157 and is more likely to be imported into the nucleus where it will act to inhibit cyclin dependent kinases contributing to inhibition of the cell cycle. This increase in nuclear p27 import would be in line with our hypothesis and support our colony assay data; showing that antiestrogen resistant LCC2 cells are inhibited by tamoxifen when exposed to ROS modifiers that reduce the oxidant state of the cell.

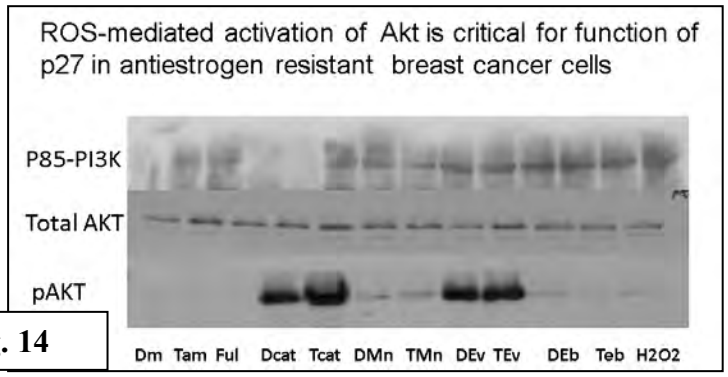
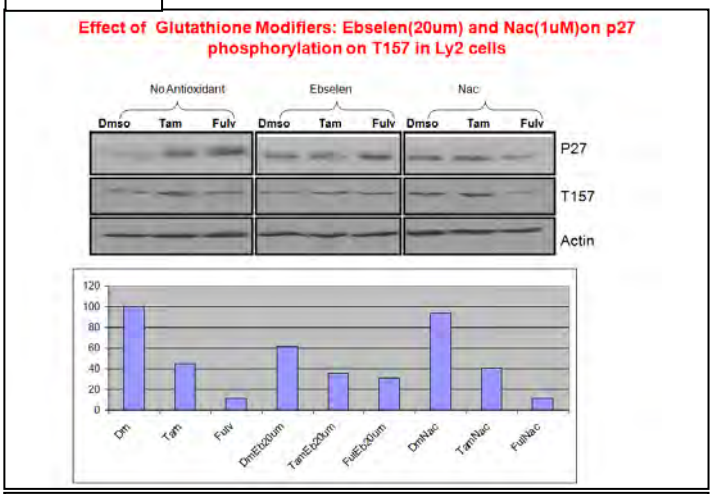
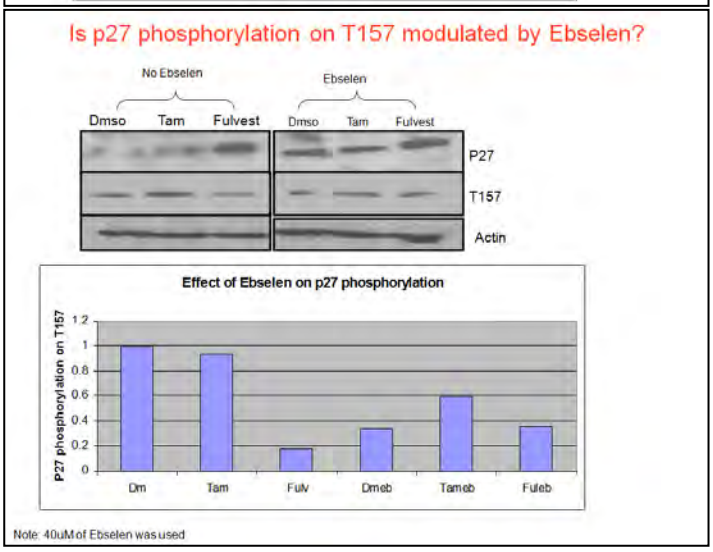


Fig. 14

8. Glutathione modifiers influenced p27 phosphorylation in antiestrogen exposed breast cancer resistant cells. It is expected that redox signaling inhibition by pharmacological and genetic approaches restores the cell cycle inhibitory function of p27, reduces phosphorylation and enhances sensitivity of resistant breast cancer tumor cells to tamoxifen or fulvestrant. These studies would confirm that adaptation to higher oxidative state in breast carcinoma is not only causally linked to antiestrogen therapy resistance, but also elucidate the mechanism thereof.

Fig. 15





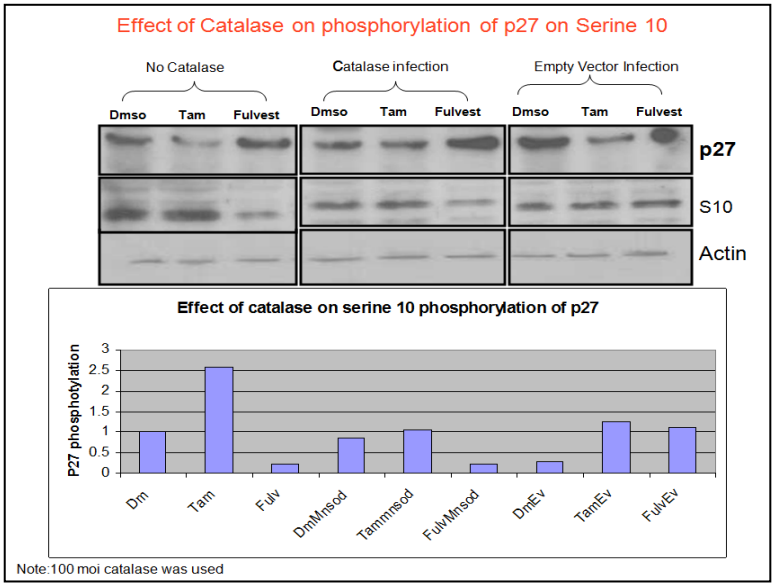
We studied the effect of GSH modifiers N-acetylcysteine (NAC) and Ebselen on the protein level of p27 in LY2 (Tamoxifen resistant) breast cancer cells exposed to antiestrogens. As shown in **Fig.** 15, both NAC and Ebselen showed a change in p27 levels compared to control. This data supports the overall hypothesis because it shows that p27 levels are restored when exposed to tamoxifen. Thus, the increase in p27 levels suggest that by decreasing the oxidant state of antiestrogen resistant breast cancer cells restores redox signaling of p27 by tamoxifen. The increase in p27 levels most likely will inhibit cyclin dependent kinase activity and ultimately contribute to the suppression of the cell cycle.

Since the activity of p27 is regulated by degradation, we also determined the effect of GSH modifiers NAC and Ebselen on phosphorylation of p27 at threonine-187. Threonine 187 phosphorylation is known to target p27 for proteolytic degradation. **The purpose of these experiments** was determine the effects of NAC and Ebselen had on p27 degradation via Thr 187. We expect to see a decrease in Thr 187 phosphorylation of p27 in cells exposed to NAC and Ebselen. As shown in **Fig. 15**, Threonine-187 levels decreased from tamoxifen exposure in LY2 cells treated with Ebselen and NAC. This correlates with the increase in p27 levels we observed in LY2 cells that regain their sensitivity to tamoxifen. The decrease in Thr-187 implies that reducing the oxidant state of the cell prevents

Thr-187 targeted degradation of p27. This increases the levels of p27 in LY2 cells exposed to tamoxifen which in turn inhibits cell cycle progression. These data partly explain the regained antiestrogen sensitivity of LY2 cells to tamoxifen via redox signaling that mediate post-translational modifications of p27 which contribute to its stability.

8. An increase in p27 Serine 10 phosphorylation in cells overexpressing catalase and/or MnSOD.

p27 is phosphorylated on many sites, including threonine 187, in vivo , with the predominant phosphorylation site being serine 10. The extent of serine 10 phosphorylation by proline-directed kinase is markedly increased in cells in the G0-G1 phase of the cell cycle compared to cells in the S or M phase. Therefore **the purpose of these experiments** was to determine whether the phosphorylation of p27 was being modulated by overexpressing catalase (an H2O2 scavenger) and MnSOD (a superoxide radical scavenger). As shown in **Fig. 16**, both catalase and MnSOD overexpressing LCC2 cells (Tamoxifen resistant) show an increase in Ser 10 phosphorylation when exposed to tamoxifen. Since Ser 10 phosphorylation is known to increase the protein stability of p27, this data supports our previous findings that p27 levels are increased reversing the antiestrogen resistance of LCC2 cells. Our data supports our hypothesis because an increase in Ser 10 of p27 would stabilize p27 protein resulting in an increase in p27 levels that would account for the regained sensitivity of antiestrogen resistant LCC2 cells have to Tamoxifen.



9. A decrease in Thr 187 phosphorylation of p27 in cells overexpressing CAT and/or MnSOD.

Since the activity of p27 is regulated by degradation, we determined the effect of CAT and MnSOD overexpression had on phosphorylation of p27 at threonine-187. Threonine 187 phosphorylation is known to target p27 for proteolytic degradation. **The purpose of these experiments** was determine the effects of CAT and MnSOD overexpression had on p27 degradation via Thr 187. As shown in **Fig. 17**, Threonine-187 levels decreased from tamoxifen exposure in LCC2 cells overexpressing CAT. This correlates with the increase in p27 levels we observed in LCC2 cells that regain their sensitivity to tamoxifen. The decrease in Thr-187 implies that reducing the oxidant state of the cell prevents Thr-187 targeted degradation of p27. This increases the levels of p27 in LCC2 cells exposed to
tamoxifen which in turn inhibits cell cycle progression. In addition, we observed a similar decrease in Thr-187 in the LCC2 cells overexpressing MnSOD (**Fig. 17**). Together these results are consistent with our previous data showing an increase of p27 in tamoxifen exposed LCC2 cells overexpressing antioxidant enzymes catalase (CAT) and MnSOD. These data partly explain the regained antiestrogen sensitivity of LCC2 cells to tamoxifen via redox signaling that mediate post-translational modifications of p27 which contribute to its stability.

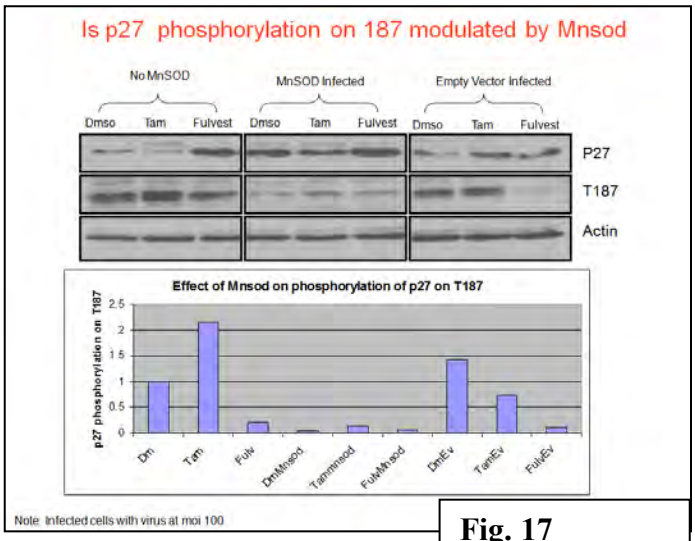


Fig. 17

10. The antiestrogen resistant breast cancer cell line LCC2 show an increase in p27 interaction with Cdk2 and cyclin E in response to tamoxifen treatment when co-treated with the ROS modifier ebselen. As shown in Fig.18, ebselen (Eb) treatment increased p27 binding to cyclin E (CyE) and Cdk2 that would impair p27 proteolysis. This is consistent with our previous finding that showed an ebselen-mediated increase in p27 levels restored tamoxifen sensitivity to antiestrogen-resistant breast cancer cell lines. In summary, these data provide further evidence for investigating ROS inhibitors in breast cancer therapy. Vehicle control is DMSO (D).

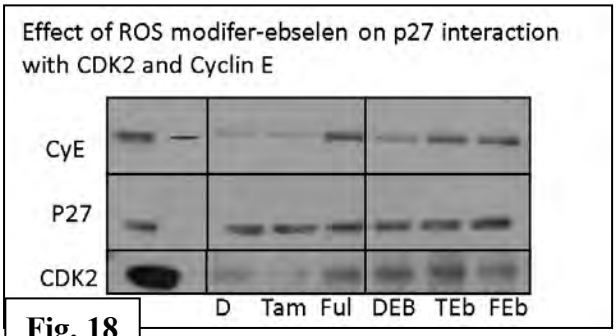


Fig. 18

We also determined the effects of specific ROS scavenging enzymes have on the protein level of p27, cyclin D, cyclin E and CDK2 in tamoxifen resistant breast cancer cells (LCC2) exposed to antiestrogen. Hydrogen peroxide was scavenged by overexpression of the enzyme catalase and superoxide radical was scavenged by overexpression of the enzyme MnSOD. We expect that overexpression of catalase and/or MnSOD will shift the intracellular redox status of resistant breast cancer cells to a more reduced-state. This shift from a pro-oxidant to a more reduced state is expected to restore the cell cycle inhibitory function of p27. In specific, the more reduced intracellular environment will restore p27's influence on cyclins (i.e. interaction of p27 with cdk2/cyclin E) which in turn will enhance the sensitivity of resistant breast cancer tumor cells to the drugs tamoxifen or fulvestrant which are known to effect p27. These studies would confirm that adaptation to a higher oxidative state in breast carcinoma is not only causally linked to antiestrogen therapy resistance, but also elucidate the mechanism thereof.We determined the effect of ROS modifiers- catalase (cat) and manganese superoxide dismutase (Mn) on the protein levels of: p27, cyclin D (CyD), cyclin E (CyE), and CDK2 in LCC2 breast cancer cells exposed to antiestrogens. As shown in Fig. 19, tamoxifen (T) or Fulvestrant (F) treated LCC2 cells overexpressing catalase or MnSOD did not show anticipated results, because the expression of these proteins was detected by Western blotting, which measures both free and bound cyclin D, E and CDK2. The cyclins bound to p27 reflects the function of p27

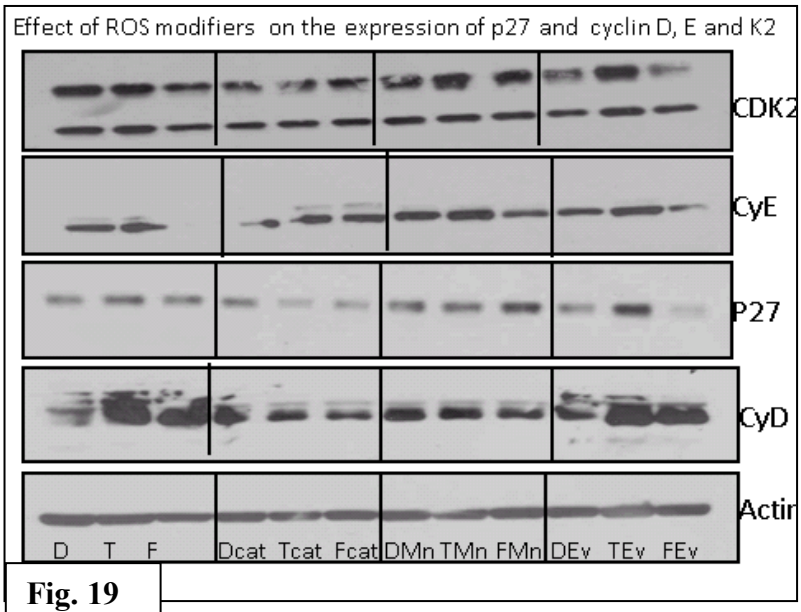


Fig. 19

KEY RESEARCH ACCOMPLISHMENTS

- Antiestrogen resistant cell lines LCC-2 and LY-2 regained sensitivity to the growth inhibitory effects of antiestrogens Tamoxifen and Fulvestrant by treatment with ROS modulators.
- Showed that the mechanism of regained antiestrogen sensitivity of LCC2 to tamoxifen is mediated by redox sensitive stability of p27.
- Showed that overexpression of ROS modifiers catalase in the tamoxifen resistant breast cancer cell line LCC2 restored sensitivity to growth inhibition by tamoxifen through activating AKT phosphorylation.
- Showed that the mechanism of regained antiestrogen sensitivity of LCC2 to tamoxifen is mediated by redox sensitive phosphorylation of p27 at Ser-10, Thr-187, and Thr-157.

REPORTABLE OUTCOMES

Publications:

Abstracts:

1. Penny, R., Felty, Q., *Slingerland, J, and Roy, D. Ebselen co-treatment counteracts the effects of antiestrogen on estrogen-induced growth of breast cancer cells as well as restores the growth inhibitory effects of antiestrogen in resistant cells. 2008 Era of Hope Meeting
2. Garba, N.A., Penny R, Okoh, V., Felty, Q., Slingerland, J*, and Roy, D. Reversible Inactivation of CDC25A by Estrogen and Antiestrogen-Induced Reactive Oxygen Species may be Involved2008 Era of Hope Meeting in the Phosphorylation of P27.
3. Penney, R., Slingerland, J., and Roy, D Redox status contributes to tamoxifen-resistant growth of breast cancer. In: CTRC-AACR San Antonio Breast Cancer Symposium in December 2009
4. Penney, R., Slingerland, J., and Roy, D. Increase in TrxR by sulforaphane contributes to tam sensitivity in tam resistant LCC2 cells by increasing expression of p27. In: Proceedings of the Annual Meeting of SOT, 2010.
5. Felty Q. Estrogenic PCBs increase blood vessel formation by redox signaling [abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr 4016.
6. Roy D, Felty Q, Okoh V. NRF-1 signaling participates in the estrogen-mediated growth of breast cancer cells. [abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr 3360.
7. Garba, N.A., Parkash, J., Felty, Q., Slingerland, J., and Roy, D Reactive oxygen species-mediated redox signaling may contribute to the development of antiestrogen resistance in breast cancer, *Era of Hope 2011 Meeting*.
8. Penney R., Felty, Q., Slingerland, J., and Roy, D. (2010). Erucin treatment through induction of thioredoxin reductase may alter tamoxifen resistance in LCC2 cells. *Amer Assoc Cancer Res. April AACR Meeting, Washington DC.*
9. Penney R., Felty, Q., Slingerland, J., and Roy, D. (2010) Modulation of thioredoxin reductase by sulforaphane may restore tamoxifen sensitivity in resistant LCC2 cells. *March SOT Meeting, Salt Lake City, UT*.

Manuscript Underpreparation

1. Nana-Aisha Garba, Quentin Felty, Joyce Slingerland, Deodutta Roy. 2011. The role of redox signaling in tamoxifen resistant breast cancer.
2. Garba NA., Roy, D. 2011. Estrogen Induced ROS mediates in vitro cell proliferation and growth through PTEN oxidation and AKT-NRF1 phosphorylation.

Grants Awarded:

1. Felty, Q. (Principal Investigator) 07/01/09-06/30/12
   Florida Department of Health, Bankhead-Coley Research Program NIR Award (09BN-06)
   Title: "Metastases and Promotion of Aggressive Angiogenic Phenotype in Breast Cancer"

PhD Awarded: Rosalind Penny, one of trainees on this grant, was awarded the degree of PhD in Spring 2011

New Faculty Hired: A new faculty (Dr. Mehmet Dorak) was added to our cancer program in the department.

CONCLUSION

Our findings show support towards our hypothesis, with increased ROS and cell proliferation in breast cancer cells upon treatment with antiestrogens, and the cotreatment with ebselen or overexpression of catalase, MnSOD or TrxR2 restore the growth inhibitory effects of antiestrogen in resistant cells. ROS modifiers restore the growth inhibitory effects of antiestrogen in resistant cells through regulating phosphorylation of p27 and interaction of p27 with Cdk2 and cyclin E.